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Jensen, Lars Bogø; Molin, Søren; Sternberg, Claus; Ramos, J.L.

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Programmed Survival of Soil Bacteria

S. Molin[1], L.B. Jensen[1], C.S. Kristensen[1], and J.L. Ramos[2]

[1]Department of Microbiology, The Technical University of Denmark, DK-2800 Lyngby, Denmark

[2]Estación Experimental del Zaidín, CSIC, Apto 419, E-18080 Granada, Spain

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Summary

Biological containment systems have been developed for *Pseudomonas putida* and related soil bacteria. The systems are based on combinations of lethal genes and regulated gene expression. Two types of killing function have been employed: 1) A membrane protein interfering with the membrane potential (*gef*), and 2) a nuclease attacking nucleic acids intracellularly. The efficacy of these lethal genes has been assessed in model constructions with a synthetic *lac* promoter. By combination with the regulatory pathway of the TOL genes, a system was designed which allows bacterial growth in the presence of aromatics but results in suicide after degradation of the compound. Time dependent induction of suicide is under construction.

1. Introduction

Although genetically engineered microorganisms (GEMs) have been considered potentially dangerous since the early days of recombinant DNA work, the contained use of them in laboratories and fermentation plants has gradually been approved and wellcomed because of the many beneficial applications in science and society. However, there still is much concern about the non-contained applications of GEMs (deliberate release), and much of this concern has to do with the lack of predictability of the fate of the released organisms and the ecological effects of their presence in the environment. We have for some time been engaged in the design of biologically contained bacteria with a higher level of predictability as far as their persistance in the environment is concerned (3).

2. The Killing Genes

The major objective of the search for suitable lethal genes for the design of biological containment systems has been to identify functions interfering with central cellular activities, which can not easily change to immunity towards the lethal function. The characteristics of the small proteins (about 50 amino acids) of the *gef* gene family fulfill these criteria since their target site in the bacteria is the cytoplasmic membrane in which they are inserted as a porin with the consequence that the membrane potential collapses followed by cell death (4,5). An analysis of

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a mutant strain of *E. coli* resistant to high levels of Gef protein showed that more than one mutation are required for this phenotype; thus, target mutations only appear with extremely low frequencies (6). Transposons carrying the *gef* gene under control of a *lac* promoter and the *laci*^{q} gene product have been constructed, and by transfer of these from *E. coli* donor strains to other gram-negative bacteria in which they integrate in the chromosomes, it has been fairly simple to analyse the inducible lethality effect of *gef* in one copy conditions. For example, *P. putida* strain KT2440 is effectively killed after induction, whereas *P. fluorescens* strain R_2f is not affected.

An almost ideal target for a containment suicide function is DNA, since removal of genetic material would be the cause of death, thus solving more than one risk problem. Other important aspects of a lethal nuclease are the lack of cellular immunity and an infinitely broad organism spectrum. We have chosen to use the extracellular nuclease of *Serratia marcescens* which has a very high specific activity against all types of nucleic acids. In *S. marcescens* the nuclease is totally excreted to the outside environment, and the sequence of the gene indicates that a consensus signal peptide is at least in part responsible for this excretion. We constructed a deletion derivative of the nuclease gene which comprises only the coding sequence for the mature polypeptide, and fused this to an expression vector carrying a *lac* promoter, a ribosome binding site and a translation start codon. Induction of the resulting non-excretable nuclease resulted in growth inhibition, DNA break-down (as judged from agarose gels with total cellular DNA), and killing of more that 99.9% of the cells. The nuclease-based containment system was introduced in soil bacteria in the same way as described for the *gef* system (transposons), and in all cases the induction of the nuclease resulted in growth inhibition. Thus, especially for strains not responding to the *gef* product it is advantageous to use the nuclease.

3. Gene Expression Control

The IPTG inducible *lac* promoter is a handy regulated expression system for testing the various killing genes in different bacteria, but in a release context the value of this type of expression system is very limited. We have, therefore, developed other combinations of lethal gene and regulated promoter which are more specifically directed towards specific applications in the environment. In all cases, however, the goal is the same: To obtain strains which are competitive (non-disabled) under the conditions of specific performance (as e.g. biopesticides, biodegraders etc.), and which will have very limited survival and proliferation potential under essentially all other environmental conditions. Two different strategies have been employed to meet this goal: 1) Stochastic induction of killing, and 2) site-specific induction of killing. In addition, we are presently working on a strategy involving killing after prolonged starvation conditions.

Stochastic induction of gene expression is illustrated by the phenomenon of phase-variation in bacteria. Pili, flagella and other cell surface components are in several cases subject to a genetic switch activation resulting in a more or less ran-

dom switch from one phenotype to another with a frequency that is significantly higher than average mutation frequencies. In case of Type 1 pili on the surface of the enterics the expression promoter responsible for the synthesis of the major pilin protein is located within a sequence of DNA which through a site specific recombinational event is either in the 'on' or the 'off' position relative to the pilin gene (2). We have found that this switch takes place in *E. coli* with a rate that is very little influenced by the growth rate, and an exchange of the pilin gene with the *gef* killing gene resulted in a strain which grows normally under conditions of excess food supplies, but under conditions of very poor growth or in stationary cells the population is continuously reduced with a rate reflecting the stochastic switch from 'off' to 'on' expression of the *gef* gene. Thus, in an environment allowing only limited growth, the bacteria with this type of stochastic killing will gradually be eliminated over a period of weeks to months.

Biodegradation of xenobiotics is an example of an environmental application of GEMs. The TOL degradation pathway for mineralization of aromatics and its regulation has been studied in detail (7), and we have exploited the extensive information available about the involved genes to design a model system of biological containment, which is founded on the concept that the degrading organism must be active and survive as long as the degradable compound is present, but after its total mineralization the bacteria should be eliminated. Such a containment principle has been achieved through insertion of the *lac* regulatory loop from *E. coli* within the regulatory pathway of the TOL genes. After having shown that the system performs as expected in *E. coli* (1), it has now been transferred to *P. putida* harboring the TOL genes, and the resulting strain was placed in soil supplemented with or without 3-methylbenzoate. The presence of 3-methylbenzoate allowed the strain to survive for a long time similar to a non-contained control strain; however, in the absence of the aromatic the contained strain was gradually eliminated from the soil in contrast to the parallel control strain. Thus, with the knowledge available about the specific regulation of the genes of interest in the release context, it is apparently possible to introduce suicide systems in such a way that the released bacteria will be confined to an area or a specific task outside which they will be disabled, and most likely rapidly competed out or totally eliminated.

4. Conclusions

Biological containment is **not** an absolute safety device. No biological containment system is totally effective, and the most serious problem is that of mutations suppressing the conditionally lethal phenotype. We do believe, however, that the significant reduction in survival potential offered by the described systems or by similar approaches may be considered to be sufficiently effective to allow more elaborate experiments to be carried out with GEMs in the open environment.

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